PB84-187368

Postnatal Developmental Alterations Following Prenatal Exposure to the Herbicide 2,4-Dichlorophenyl-p-nitrophenyl Ether: A Dose Response Evaluation in the Mouse

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1983

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. REC PB84-187368
EPA-600/J-82-177		

4. TITLE AND SUBTITLE
Postnatal Developmental Alterations following Prenatal Exposure to the Herbicide 2,4-Dichlorophenyl-p-nitrophenyl Ether: A Dose Response Evaluation in the Mice

5. REPORT DATE
1983

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S) (Journal Version)
E. Gray, R. Kavlock, N. Chernoff, J. Ostby and J. Ferrell

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Developmental Biology Division
Health Effects Research Laboratory
USEPA
Research Triangle Park, NC 27711

10. PROGRAM ELEMENT NO.
ACSL1A

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
Office of Research & Development
Health Effects Research Laboratory
USEPA
Research Triangle Park, NC 27711

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA-600/11

15. SUPPLEMENTARY NOTES
REF: Toxicology and Applied Pharmacology 67: 1-14, 1983

16. ABSTRACT

Although nitrogen, 2,4-dichlorophenyl-p-nitrophenyl ether, is a relatively nontoxic herbicide, prenatal exposure to doses considerably less than the LD50 value for rats and mice produces numerous developmental defects that become apparent as the animals mature. In the present study postnatal development was observed following prenatal exposure during Days 7 to 17 of gestation at doses of 0, 6.25, 12.5, 25, 50, 100, 150, and 200 mg/kg/day. These doses did not cause maternal toxicity as indicated by the viability of the dams or maternal weight gain during pregnancy. By 3 days of age all pups in the two highest dose groups were dead and 50% had died in the 100 mg/kg/day dose group. Some of the dead and moribund pups from the 200 mg/kg/day exposure group necropsied at three days of age had cleft palate (15%) or diaphragmatic hernia (6%). In addition, about 22% of the pups at 200 mg/kg/day developed a distended abdomen from gasping and swallowing air. These pups did not suckle and eventually died. Body weights of offspring were reduced at birth in the 150 and 200 mg/kg/day groups and at 3 days of age in the 100 mg/kg/day group. Growth rates were subsequently retarded at 12.5, 25, 50, and 100 mg/kg. The Harderian glands were reduced or absent in 97, 65, and 4% of the mice in the 100, 50, and 25 mg/kg dosage groups, respectively, and the gland weights were reduced at all dosages, including the lowest dose of 6.25 mg/kg/day.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
		15
	20. SECURITY CLASS *(This page)*	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

REPRODUCED BY
NATIONAL TECHNICAL INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

EPA-600/J-82-177
JOURNAL ARTICLE

TOXICOLOGY AND APPLIED PHARMACOLOGY 67, 1–14 (1983)

Postnatal Developmental Alterations following Prenatal Exposure to the Herbicide 2,4-Dichlorophenyl-*p*-nitrophenyl Ether: A Dose Response Evaluation in the Mouse

L. EARL GRAY, JR.,[1] ROBERT J. KAVLOCK, NEIL CHERNOFF, JOE OSTBY, AND JANET FERRELL

Developmental Biology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

Received April 19, 1982; accepted October 6, 1982

Postnatal Developmental Alterations following Prenatal Exposure to the Herbicide 2,4-Dichlorophenyl-*p*-nitrophenyl Ether: A Dose Response Evaluation in the Mouse. GRAY, L. E., JR., KAVLOCK, R. J., CHERNOFF, N., OSTBY, J., AND FERRELL, J. (1983). *Toxicol. Appl. Pharmacol.* 67, 1–14. Although nitrofen, 2,4-dichlorophenyl-*p*-nitrophenyl ether, is a relatively nontoxic herbicide, prenatal exposure to doses considerably less than the LD_{50} value for adult rats and mice produces numerous developmental defects that become apparent as the animals mature. In the present study postnatal development was observed following prenatal exposure during Days 7 to 17 of gestation at doses of 0, 6.25, 12.5, 25, 50, 100, 150, and 200 mg/kg/day. These doses did not cause maternal toxicity as indicated by the viability of the dams or maternal weight gain during pregnancy. By 3 days of age all pups in the two highest dose groups were dead and 50% had died in the 100 mg/kg/day dose group. Some of the dead and moribund pups from the 200 mg/kg/day exposure group necropsied at three days of age had cleft palate (15%) or diaphragmatic hernia (6%). In addition, about 22% of the pups at 200 mg/kg/day developed a distended abdomen from gasping and swallowing air. These pups did not suckle and eventually died. Body weights of offspring were reduced at birth in the 150 and 200 mg/kg/day groups and at 3 days of age in the 100 mg/kg/day group. Growth rates were subsequently retarded at 12.5, 25, 50, and 100 mg/kg. The Harderian glands were reduced or absent in 97, 65, and 4% of the mice in the 100, 50, and 25 mg/kg dosage groups, respectively, and the gland weights were reduced at all dosages, including the lowest dose of 6.25 mg/kg/day. Weights of other organs including lung and liver (at 6.25 and above), seminal vesicle (at 12.5 and above), and testes (at 100 mg/kg/day) were also reduced by prenatal nitrofen exposure. In addition, prenatal treatment with nitrofen produced functional deficits of the reproductive system; puberty was delayed in females and litter sizes were reduced at 50 and 100 mg/kg/day. A cross-fostering experiment with 100 mg/kg/day of nitrofen demonstrated that the effects noted in the present study were produced solely by prenatal exposure; pups exposed to nitrofen in the milk alone as a consequence of any accumulation of nitrofen in the dam during gestation were unaffected.

Nitrofen [2,4-dichlorophenyl-*p*-nitrophenyl ether] is a herbicide for garden and vegetable crops. Although nitrofen is relatively nontoxic, having an LD_{50} in adult rats and mice greater than 1000 mg/kg (Ambrose *et al.*, 1971), administration of nitrofen during pregnancy at dose levels two to three orders of magnitude below the LD_{50} causes diaphragmatic hernias, retards lung development (Stone and Manson, 1981; Nakao *et al.*, 1981; Costlow and Manson, 1981), and reduces survival after birth (Ambrose *et al.*, 1971; Kimbrough *et al.*, 1974; Chernoff and Kavlock, 1980). In studies in our laboratory with mice, the survivors exposed to nitrofen at 200 mg/kg/day by oral gavage on Days 8 to 12 of

[1] To whom all correspondence should be addressed.

0041-008X/83/010001-14$03.00/0
Copyright © 1983 by Academic Press, Inc.
All rights of reproduction in any form reserved.

gestation are grossly deformed at weaning (Gray *et al.*, 1982). While these abnormalities are extensive and include hydrocephaly, hyperactivity, malocclusion of the jaws, and anophthalmia, none of these defects, including the anophthalmia, are apparent in newborn mice. We have determined that the subsequent anophthalmia is due to alteration of the Harderian glands. The Harderian gland defect is present in newborn mice but the eyes do not appear abnormal until eye opening because the Harderian glands do not begin to function until this time. In the treated mice, eye opening is delayed as much as 20 days and the palpebral fissures are reduced causing mice to appear microphthalmic. The Harderian gland defect was also observed in rats exposed *in utero* to nitrofen at 12.5 mg/kg/day on Days 8 to 16 of gestation.

The present study was designed to describe the dose–response relationship of nitrofen to the Harderian gland defect in the mouse and to compare the sensitivities of other affected organ systems. In the present study mice were exposed to nitrofen during gestation and examined after birth. The gross morphology, growth, viability, reactive locomotry activity, the age at puberty, fertility, and fecundity were measured and the Harderian glands, lungs, and other organs were examined and weighed.

METHODS

General. Primiparous mice, *Mus musculus* (CD-1), shipped on Day 5 of gestation from Charles River Breeding Laboratories (Wilmington, Mass.), were housed individually in transparent plastic cages (33 × 20 × 13 cm) with pine shavings as litter. Cages were changed three times per week. All animals were maintained on a 12:12 light:dark photoperiod with the lights going out at 1800 (EST). The animals were given Wayne Lab Blox and tap water *ad libitum*. Upon arrival dams were randomly assigned to control or treated groups and dosed by gavage with nitrofen in corn oil (volume 0.5 ml) on Days 7 to 17 of gestation.

The first experiment was conducted in two blocks. In the first block, dams were dosed with 0, 50, 100, 150, or 200 mg/kg/day of nitrofen (99.6% pure) on Days 7 to 17 of gestation. A teratology study was conducted concurrently with this postnatal experiment. Day 19 fetal mice were preserved in alizarin or Bouin's solution for examination of skeletal and soft tissue defects, respectively. In the postnatal study, there were 19, 17, 14, 14, and 11 dams with viable litters on Day 1 at 0, 50, 100, 150, and 200 mg/kg/day nitrofen, respectively. Litter sizes and weights were determined on postnatal Days 1 and 3. At this time dead or dying pups were taken and preserved in Bouin's solution. These pups were necropsied and examined for gross morphological defects by methods similar to those used in the examination of the fetal mice in the teratology study (Courtney and Chernoff, 1974).

At 22 to 24 days of age the diurnal reactive locomotor activity levels of 18 (0 mg/kg/day), 10 (50 mg/kg/day), and 11 (100 mg/kg/day) randomly selected male and female mice were measured for 1 hr in figure 8 mazes (Reiter *et al.*, 1977).

At 30 days of age the pups were weaned, counted, and weighed, and the number of females with patent vagina was determined as an index of puberty.

In the second block, the doses of nitrofen were lower than those in Block 1; 0, 6.25, 12.5, 25.0, and 50 mg/kg/day. There were 23, 19, 27, 21, and 17 litters, respectively, on Day 1.

On Day 7, eight, six, eight, eight, and eight litters were randomly selected and maintained until weaning from the 0, 6.25, 12.5, 25, and 50 mg/kg/day dose groups. Pups were counted and weighed on Days 7 and 30; the percentage of females with patent vaginas was determined on Day 30, and the percentage of pups per litter with obviously reduced palpebral fissures was also determined at this time. These data were analyzed by litter means. At 35 days of age, 16 breeding pairs per dose group were established, and the surplus males were necropsied. The height of the palpebral fissures, body weight, and weights of the eyes, lungs, testes, seminal vesicle, and kidney were determined. The breeding pairs were checked daily from 50 days of age throughout the experiment. When pups were born they were removed, and the age of the breeding pair and the number of pups were recorded. The males from the breeding pairs were necropsied at 110 days of age, and body weight and weights of the lungs, Harderian glands, testes, seminal vesicle, kidney, and liver were measured. At 130 days of age, following the delivery of the last litter, the females were necropsied and body, lung, and Harderian gland weights were determined. In the second block locomotor activity was not measured because it was unaffected at higher doses in Block 1.

A cross-fostering experiment was conducted concurrently with the first block of Experiment 1. In this experiment, dams were dosed with the vehicle (CON) or nitrofen (N) at 100 mg/kg/day as in Experiment 1. At birth all pups were cross-fostered to a new dam with one-half of the litters from CON and N dams being assigned to dams in the other treatment group (CON/N and N/CON) while the other half of the litters were assigned to dams in the same treatment group (CON/CON and N/N). On Day 1 there were 13 (CON/CON—prenatal/post-

natal), 13 (CON/N), 12 (N/CON), and 14 (N/N) litters per group. The growth and viability of these pups were observed at 1, 3, 10, and 20 days of age.

At 3, 8, and 13 days of age six control and six prenatally treated pups were preserved in Bouin's solution, and coronal sections were made to describe the development of the Harderian gland.

At 35 days of age males and females from the same dose group but from different litters were housed in breeding pairs until the termination of the experiment. The breeding data were collected as in Experiment 1.

At 110 days of age the male mice were killed and necropsied. The body weight, lung weight, weight of both Harderian glands, testes, and seminal vesicles were recorded. Mice were killed with CO_2 in these studies, a method which causes a 10% increase in the lung weight of mice.

Statistical methods. The data were analyzed by the General Linear Models (GLM) procedure available on the Statistical Analysis System (SAS) (Helwig and Council, 1979). The preweanling data on offspring growth and viability were analyzed by a one-way (DOSE) analysis of variance (ANOVA). The postweanling data were initially analyzed as 5 (doses of nitrofen) × 2 (sexes when appropriate) × 2 (ages when appropriate) factorial design with incomplete blocks. The linear component of the dose effect was determined, and any organ weights significantly altered by nitrofen were reanalyzed with body weight as a covariate to determine if organ weight reductions were related to the reduced body weight. The cross-fostering data were analyzed as a 2 (prenatal treatment) × 2 (postnatal treatment) factorial. Statistical significance was determined by the partial sums of squares rather than the sequential sums of squares due to unequal sample sizes. Post hoc tests were made by a t test when the ANOVA indicated that a treatment affect existed.

RESULTS

Dose–Response Experiment

Maternal effects. The administration of nitrofen did not cause any adverse effects in the dams as measured by maternal viability or weight gain from Days 7 to 19 of gestation (Table 1).

Offspring effects. Nitrofen significantly reduced the number of live pups on Day 1 at 150 mg/kg/day (20%) and at 200 mg/kg/day (30%) (Table 1). By Day 3 there were no live pups at these higher doses, and the number of pups was reduced significantly at 100 mg/kg/day (55%) and at 50 mg/kg/day (20%).

Unlike the results of earlier studies, which used higher doses of nitrofen for a shorter period during gestation, offspring in the present study did not develop hydrocephaly, malocclusion of the jaws, or anophthalmia as they matured.

Body weights were permanently reduced from Days 1 and 3 at doses of 50 mg/kg/day and above of nitrofen (Tables 1 and 2). In block 2, body weights at these ages were unaffected by nitrofen at doses below 50 mg/kg/day but an effect of nitrofen on growth became apparent and was significant at 12.5 mg/kg/day and above as the offspring matured (Table 3).

An examination of the gross morphology of dead and dying pups recovered at 1 and 3 days of age indicated that the brain, eyes, heart, lungs, liver, and digestive system were unaffected by prenatal nitrofen treatment. A gross examination of the Harderian gland is not possible because of their small relative size at these ages.

Nitrofen treatment produced lethal defects in the pups (Table 1). There were increases in the frequency of cleft palate, a defect not previously noted to occur with nitrofen, and diaphragmatic hernias (previously noted by Nakao *et al.*, 1981). In addition, another condition, a "Ballooning Syndrome," was noted in the pups. This condition was usually fatal and was not consistently associated with any other obvious abnormality. Pups with this syndrome would gasp and swallow air which eventually filled the stomach and intestines, causing extreme abdominal distention (ballooning). These pups did not suckle and eventually died.

We used the incidence of these three lethal defects to estimate the frequency of abnormalities in each dosage group. In order to do this estimation, we assumed that (1) the recovered pups represented a random sample of the population of all the pups that died in that dosage group and (2) that pups did not recover from these abnormalities. The (estimate incidence of these defects) = (observed incidence in the sampled population) × (the % mortality). It is important to note that only 42% of the deaths at 200 mg/kg/day can be

TABLE 1

MATERNAL AND NEONATAL EFFECTS OF NITROFEN ADMINISTERED ORALLY ON DAYS 7 TO 17 OF PREGNANCY IN BLOCKS 1 AND 2

Dose (mg/kg/day)	0	6.25	12.5	25	50	100	150	200
Maternal effects								
No. treated	54	29	31	29	41	20	25	29
No. died	3	4	0	2	0	2	1	6
No. resorbed	1	1	0	1	0	0	0	1
No. late	2	1	0	1	0	0	5	3
No. not pregnant	7	5	4	5	6	4	5	8
No. pregnant at term	41	18	27	20	35	14	14	11
Weight gain[a,b] (g)	4.6	5.3	4.9	5.8	4.8	4.9	5.5	4.8
Neonatal effects								
No. live								
Day 1[b]	10.7	10.0	11.6	10.7	10.6	9.6	8.7[c]	7.6[d]
Day 3	10.4	9.4	11.0	10.7	8.7[d]	4.2[d]	0.1[d]	0.2[d]
Pup weight (g)								
Day 1	1.62	1.59	1.58	1.58	1.51[d]	1.40[d]	1.40[d]	1.33[d]
Day 3	2.02	2.06	2.03	1.99	1.85[d]	1.53[d]	1.22[d]	1.18[d]
No. dead pups recovered and necropsied	6	0	7	5	26	50	118	72
Total affected (%)	17	—	0	0	12	26	31	43
Cleft palate (%)	17	—	0	0	4	2	14	15
Diaphragmatic hernia (%)	0	—	0	0	0	0	0	6
"Ballooning" syndrome (%)	0	—	0	0	8	24	19	22
Estimated totai defects in population[e] (%)	0.48	—	0	0	2.2	14.6	30.6	41.9

[a] Maternal body weight (g) change from Days 7 to 19 of gestation. Including only those female pregnant at term.
[b] Values are means.
[c] $p < 0.05$, different from control mean.
[d] $p < 0.01$, different from control mean.
[e] Estimated % of defects = (% mortality) × (% dead affected).

TABLE 2

THE EFFECTS OF PRENATAL EXPOSURE TO NITROFEN ON THE POSTNATAL DEVELOPMENT OF CD-1 MICE

	Dose of nitrofen (mg/kg/day)		
	0	50	100
Body weight day 30 (g)	25.1 ± 0.4 (10)[a]	22.4 ± 0.4 (8)[b]	20.8 ± 1.0 (4)[b]
Percentage of females with patent vaginas on Day 30 (Puberty)	90 ± 7 (10)[a]	65 ± 16 (8)	21 ± 12 (4)[b]
Figure 8 maze locomotor activity (counts/60 min)	648 ± 44 (19)[c]	579 ± 21 (11)	597 ± 23 (18)

Note. Dams were dosed by gavage on Days 7 to 17 of gestation.
[a] Values are $\bar{X}$ ± SE (No. of litters).
[b] $p < 0.01$.
[c] Values are $\bar{X}$ ± SE (No. of individuals).
Data are from the first block of the dose response experiment.

TABLE 3

THE EFFECTS OF PRENATAL NITROFEN ADMINISTRATION ON POSTNATAL GROWTH, VIABILITY, AND NONREPRODUCTIVE ORGAN WEIGHTS OF THE MICE IN BLOCK 2

Variable	Age and sex	Dose of nitrofen (mg/kg/day) 0	6.25	12.5	25	50
No. live pups	Day 7	9.0 ± 0.7 (23)[a]	9.2 ± 0.8 (19)	8.9 ± 0.9 (27)	9.0 ± 1 (21)	8.1 ± 0.7 (17)
	Day 30	8.0 ± 0.9 (8)[a]	7.5 ± 1.4 (6)	7.9 ± 1.1 (8)	7.8 ± 1 (8)	8.0 ± 0.9 (8)
Body weight (g)	Day 7	3.6 ± 0.18 (23)[a]	3.9 ± 0.16 (19)	3.8 ± 0.14 (27)	3.7 ± 0.11 (21)	3.7 ± 0.21 (17)
	Day 30	21.8 ± 0.7 (8)[a]	21.5 ± 0.9 (6)	19.7 ± 0.9 (8)[b]	19.3 ± 0.8 (8)[c]	19.3 ± 0.8 (8)[c]
	Day 35 M	28.7 ± 0.7 (22)[d]	28.9 ± 0.6 (4)	28.1 ± 0.5 (10)	27.4 ± 0.5 (8)	26.9 ± 0.6 (18)[b]
	Day 110 M	42.1 ± 1 (16)[d]	39.4 ± 1 (16)	38.4 ± 0.8 (16)	39.0 ± 0.8 (16)	37.5 ± 1.1 (15)
	Day 110 F	38.2 ± 1.1 (16)[d]	38.0 ± 1 (16)	35.8 ± 0.7 (16)	35.2 ± 0.7 (16)	35.2 ± 1.1 (15)
	Day 110 M&F	40.4 ± 0.8 (32)[d]	38.7 ± 0.7 (32)	37.2 ± 0.6 (32)[c]	37.1 ± 0.7 (32)[c]	36.4 ± 0.8 (30)[c]
Lung weight (mg)	Day 35 M	281 ± 20 (21)[d]	234 ± 17 (4)	248 ± 19 (10)	211 ± 16 (8)[c]	198 ± 7.3 (24)[c]
	Day 110 M	270 ± 16 (16)[d]	245 ± 12 (16)	239 ± 17 (16)	247 ± 17 (16)	226 ± 11 (15)
	Day 110 F	210 ± 7 (16)[d]	208 ± 8 (16)	196 ± 7 (16)	211 ± 13 (16)	185 ± 10 (15)
	Day 110 M&F	241 ± 10 (32)[d]	227 ± 8 (32)	218 ± 10 (32)	229 ± 11 (32)	205 ± 8 (30)[c]
	Day 35 & 110 M&F	250 ± 8 (43)[d]	220 ± 14 (36)[b]	222 ± 10 (42)[b]	209 ± 11 (40)[c]	191 ± 8 (54)[c]
Kidney weight (mg)	Day 35 M	255 ± 8 (11)[d]	—	—	—	239 ± 10 (12)
	Day 110 M	371 ± 15 (9)[d]	364 ± 15 (10)	355 ± 15 (10)	367 ± 15 (9)	350 ± 15 (9)
	Day 35 & 110	313 ± 9 (20)[d]	—	—	—	295 ± 9 (21)
Liver weight (mg)	Day 110 M	2778 ± 109 (9)[d]	2452 ± 102 (10)[b]	2393 ± 103 (10)[c]	2544 ± 108 (9)	2314 ± 108 (9)[c]

Note. Dams were dosed by gavage with nitrofen on Days 7 to 17 of gestation.

[a] Values are $\bar{X}$ ± SE (No. of litters).

[b] $P < 0.05$.

[c] $P < 0.01$.

[d] Values are $\bar{X}$ ± SE (No. of individuals).

TABLE 4

THE EFFECTS OF PRENATAL NITROFEN EXPOSURE ON THE POSTNATAL REPRODUCTIVE DEVELOPMENT OF MALE AND FEMALE MICE IN BLOCK 2

	Dose of nitrofen (mg/kg/day)				
	0	6.25	12.5	25	50
Percentage of females with vaginal opening, Day 30[a]	49 ± 14 (7)	16 ± 12 (5)	28 ± 9 (7)	31 ± 12 (8)	3 ± 3 (8)[b]
Fecundity of offspring[c]					
1st litter size	11.3 ± 1.1 (14)	11.9 ± 1.1 (14)	11.9 ± 0.9 (18)	11.3 ± 0.6 (15)	10.7 ± 0.8 (11)
2nd litter size	15.3 ± 0.7 (6)	13.6 ± 0.7 (8)	13.8 ± 0.6 (10)	14.2 ± 0.6 (6)	12.0 ± 0.8 (4)[b]
1st & 2nd litter size	26.6	25.5	25.7	25.5	22.7
Testes weight[c] (mg)					
Day 35	175 ± 8 (11)	—	—	—	159 ± 6 (12)
Day 110	264 ± 10 (16)	267 ± 11 (16)	257 ± 6 (16)	244 ± 7 (16)	244 ± 7 (15)
Days 35 & 110	220 ± 6 (27)	—	—	—	204 ± 6 (27)
Seminal vesicle weight[c] (mg)					
Day 35	105 ± 9 (11)	—	—	—	87 ± 7 (12)
Day 110	418 ± 19 (16)	391 ± 15 (16)	355 ± 19 (16)[d]	378 ± 21 (16)	345 ± 19 (15)[b]
Days 35 & 110	261 ± 13 (27)	—	—	—	217 ± 13 (27)[b]

Note. Dams were dosed with nitrofen on Days 7 to 17 of gestation.
[a] Values are $\bar{X}$ ± SE (No. of litters).
[b] $p < 0.01$.
[c] Values are $\bar{X}$ ± SE (No. of individuals).
[d] $p < 0.05$.

explained and only 20% can be attributed to a specific abnormality even though all of these pups died.

Growth of the lungs was significantly reduced by nitrofen treatment at all doses including the lowest dose of 6.25 mg/kg/day (Table 3). In addition, statistical analyses indicated that the effect was greater at 35 days of age than at 110 (significant dose by age interaction $p < 0.05$). Adjusting the values for differences in body weight by analysis of covariance (AC) did not abolish the treatment effect on lung weight.

Testes weights were not significantly reduced by nitrofen treatment at the 50 mg/kg/day dose group or below (Table 4).

Seminal vesicle (SV) weights were significantly reduced by nitrofen treatment at 12.5 and 50 mg/kg/day (Table 4). AC indicated that this treatment effect was highly correlated with the treatment effect on body weight since the partial correlation of treatment and SV weight, adjusted for body weight, was not significant.

Liver weights in Block 2 were significantly reduced at 6.25, 12.5, and 50 mg/kg/day. The partial correlation of treatment to liver weight was not significant following correction for body weight by AC (Table 3).

Kidney weights were unaffected by treatment (Table 3). In addition, the kidneys were sliced through the midhilar region and examined at necropsy and they were not hydronephrotic.

Obvious intraorbital defects were present in some offspring in the 25 mg/kg/day dosage group and above. The eyes appeared microphthalmic and the palpebral fissures were

TABLE 5

INTRAORBITAL DEFECTS FOLLOWING PRENATAL EXPOSURE TO NITROFEN IN MICE

	Dose of nitrofen (mg/kg/day)				
	0	6.25	12.5	25	50
Percentage of pups with obviously reduced palpebral fissures on Day 30	0	0	0	3	36.8[a]
Palpebral fissure height on Day 35 (mm)	2.83 ± 0.09 (16)[b]	2.35 ± 0.14 (4)	2.66 ± 0.07 (10)	2.52 ± 13 (6)[c]	2.10 ± 0.07 (19)[a]
Unilateral eye weight on Day 35 (mg)	21.6 ± 0.4 (17)	21.1 ± 0.5 (4)	21.8 ± 0.3 (9)	20.6 ± 0.5 (7)	20.6 ± 0.7 (24)
Percentage of offspring with bilateral Harderian glands	100 (27)	100 (30)	100 (31)	94 (32)	35 (29)[a]
Weight (mg) of paired Harderian glands (if both were present)					
Day 110 males	41.9 ± 1.1 (16)	38.7 ± 1.1 (16)[a]	36.8 ± 1.1 (16)[a]	31.9 ± 0.9 (16)[a]	28.8 ± 1.8 (6)[a]
Day 110 females	35.5 ± 1.5 (11)	31.0 ± 0.8 (14)[a]	30.4 ± 0.7 (15)[a]	27.0 ± 0.5 (14)[a]	23.2 ± 1.5 (3)[a]

Note. Data from Block 2.
[a] $p < 0.01$.
[b] Values are $\bar{X}$ ± SE (No. of individuals).
[c] $p < 0.05$.

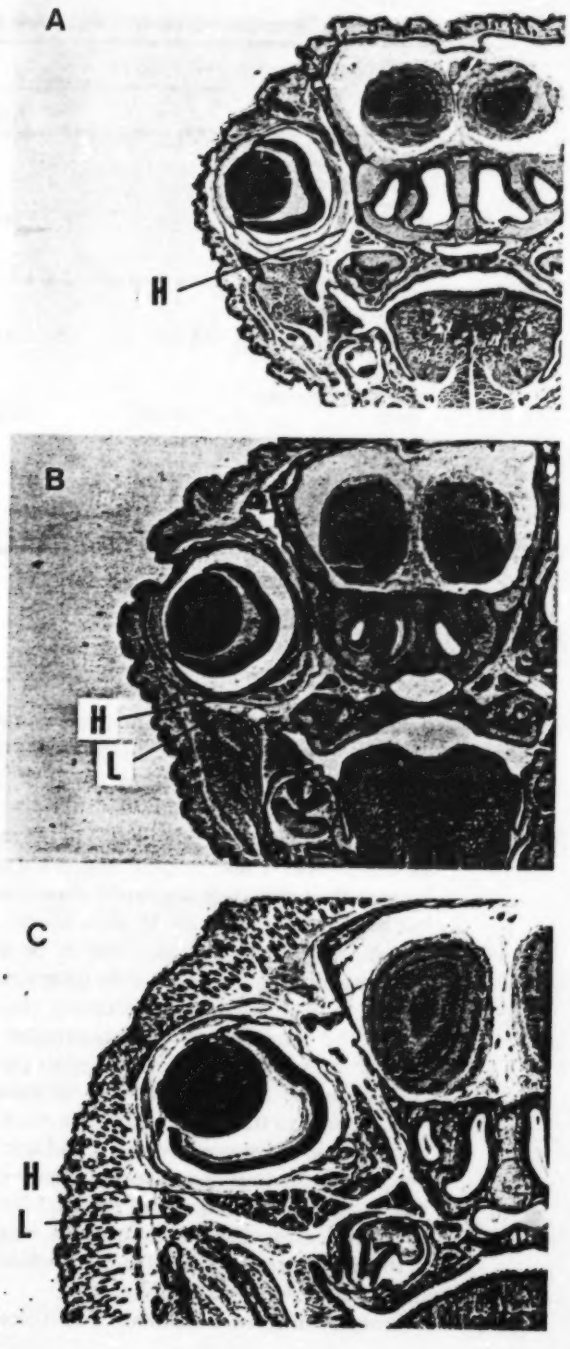
reduced, but the eyes were not small as a result of prenatal nitrofen exposure (Table 5). We initially thought that some (10%) of the pups in the 100 mg/kg/day dose (Block 1) group and a few pups (about 2%) at 50 mg/kg/day (Blocks 1 and 2) were anophthalmic because their eyes were shut at 30 days of age but their eyes opened by 35 days of age. It was apparent that what appeared to be microphthalmia was the result of the destruction or reduction in size of the Harderian glands behind the eyes (Fig. 1). An examination of Fig. 1 demonstrates that the Harderian gland is small in the 3-, 8-, and 13-day-old mouse as compared to the adult, and the absence of this gland in the treated animal could not be detected in the fetal or neonatal mouse without microscopic examination. Prenatal nitrofen treatment significantly reduced the weight of the Harderian glands at all doses, including the lowest dose of 6.25 mg/kg/day.

Prenatal nitrofen treatment of 50 (Blocks 1 and 2) and 100 mg/kg/day significantly delayed puberty in the female mice as indicated by the percentage of females with patent vaginas on Day 30 (Tables 2 and 4). In addition, the 50 mg/kg/day females, paired with treated males, produced significantly fewer pups in the second litter (Table 4).

The 60-min reactive locomotory activity levels of the 22- to 24-day-old mice were unaffected by prenatal nitrofen treatment (Table 1).

Cross-Fostering Experiment

The results of the cross-fostering experiment are shown in Table 6. In summary, all of the effects noted in the present study were the result of prenatal exposure to nitrofen, with (N/CON) and (N/N) being severely affected as compared to the (CON/N) and (CON/CON) groups. If any postnatal nitrofen


A
H
B
H
L
C
H
L

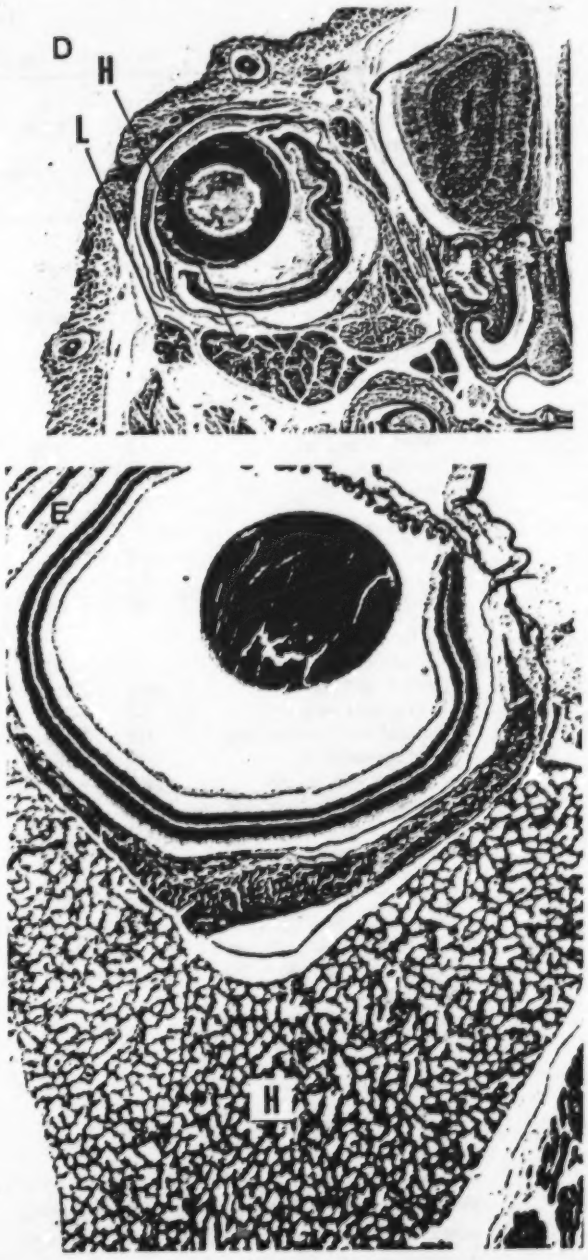


FIG. 1. This series of pictures shows the development of the Harderian gland behind the eye of the mouse, and the effect of prenatal exposure to nitrofen at 100 mg/kg/day on Days 7 to 17 of gestation on this gland. (A) A 3-day treated mouse without a Harderian gland (8.5×). (B–D) 3-, 8-, and 13-day-old untreated mice, respectively (8.5×). (E) Adult mouse (17×). H identifies the Harderian glands and L identifies the intraorbital lacrimal glands.

TABLE 6

RESULTS OF THE CROSS-FOSTERING STUDY

Group:	A	B	C	D	A + B	C + D	A + C	B + D
Prenatal:	CON	CON	N	N	CON	N		
Postnatal:	CON	N	CON	N			CON	N
No. of litters:	13	13	12	14	26	26	25	27
Viability								
No. live Day 1	11.0[a]	10.5	9.1	9.4	10.7	9.4[b]	10.0	9.9
No. live Day 3	11.0	10.3	5.7	6.1	10.7	5.9[c]	8.3	8.2
No. live Day 10	10.5	9.8	3.3	3.4	10.1	3.4[c]	6.9	6.6
No. live Day 20	10.1	9.3	3.0	2.7	9.7	2.9[c]	6.5	6.0
Percentage of pups with ballooning syndrome	0	0	28	32	0	30[c]	14	16
Growth								
Weight Day 1 (g)	1.69	1.72	1.50	1.52	1.70	1.51[c]	1.60	1.62
Weight Day 3 (g)	2.33	2.10	1.61	1.64	2.21	1.63[c]	1.97	1.87
Weight Day 10 (g)	4.91	4.76	4.51	3.52	4.84	4.01[d]	4.71	4.14
Breeding data								
F_{2A}	12.3	13.1	9.4	10.4	12.7	10.0[d]	10.9	11.7
F_{2B}	15.3	14.2	12.1	13.9	14.9	13.0[d]	13.6	14.0
No. of litters in F_{2A}/F_{2B}	9/7	9/4	11/8	12/8	18/11	23/16	20/15	25/12
110 Day necropsy: No. males necropsied	11	10	11	13	21	24	22	23
Body weight (g)	39.9	38.8	33.2	34.0	39.4	33.6[c]	36.4	36.3
Paired testes weight (mg)	281	278	225	234	280	229[d]	253	256
Seminal vesicle weight (mg)	382	362	320	293	372	305[d]	351	323
Lung weight (mg)	226	202	174	195	214	185[d]	200	198
Single eye weight (mg)	27.1	25.5	26.0	28.7	26.3	26.8	26.5	27.6
Single Harderian gland weight (mg) (if present)	21.1	20.0	—	12.4[e]	20.6	12.4[e]	21.1	19.0
Percentage with paired Harderian glands present	100	100	0	0	100	0	50	50

Note. Dams were dosed orally with nitrofen at 100 mg/kg/day or oil on Days 7 to 17 of gestation. Half of the litters in each prenatal treatment were cross-fostered to dams in the other treatment (CON/N; N/CON) while the other half were fostered to dams with the same treatment (CON/CON; N/N).

[a] Values are means.

[b] $p < 0.05$ by a t test.

[c] $p < 0.001$ by a t test.

[d] $p < 0.01$ by a t test.

[e] This value is from one gland.

exposure occurred as a consequence of consumption of the milk from dams dosed during gestation, it was without effect. In the present experiment the number of live pups was reduced by prenatal nitrofen exposure and further reduced by Day 3. In males, body weights were reduced by treatment throughout the experiment, and testes, seminal vesicle, and lung weights were lower at necropsy. The prenatally treated offspring also had smaller litter sizes (the F_{2A} and F_{2B}). None of the 24 prenatally treated males that were necropsied had paired Harderian glands. Although the mean eye weight was normal in the treated group,

the individual values were extremely variable with some of the eyes being very small and others quite large suggesting that some of the eyes were not normal.

DISCUSSION

Results of the present study demonstrate that prenatal exposure to the herbicide nitrofen produces a wide variety of developmental defects in the mouse. The Harderian glands were more severely affected by prenatal nitrofen than were the other organs measured. These glands were not detectable in 65% of the mice from the 50 mg/kg dose group and in 4% at 25 mg/kg. Nitrofen did not induce perinatal mortality in the 25 mg/kg dose group or in the lower dose groups, and although many other measured variables were affected at this dose and at lower doses, these differences were quantitative rather than qualitative.

The effect of nitrofen on the Harderian glands noted in the present study is unusual in that a macroscopic examination of the eyes of fetal (unpublished data) and neonatal mice in the present study revealed no abnormalities until after eye-opening when they appeared microphthalmic (Gray *et al.*, 1982). However, the eyes were not consistently smaller in the treated mice. Further investigation revealed that the apparent microphthalmia was due to either a reduction in size or absence of the Harderian glands in the nitrofen-treated mice causing the eyes to sink into the orbits. This intraorbital effect was not overtly apparent until 12 to 13 days of age at which time the Harderian glands grow rapidly and begin to secrete porphyrins and phospholipids (Wetterberg *et al.*, 1970). The rapid growth of the Harderian glands at this age correlates ontogenetically with a developmental increase in thyroxine secretion (Dohler *et al.*, 1979). Thyroidectomy causes Harderian gland regression (Boas and Scow, 1954), and neonatal treatment with thyroxine accelerates development of the Harderian glands (Wetterberg *et al.*, 1970).

The Harderian gland is a large bilobed, saccular exocrine gland located in the orbit of reptiles, birds, and some mammals, but is rudimentary or lacking in man (Kennedy, 1970). This organ has a single duct that empties onto the inner surface of the nicitans, and its primary function is generally designated as the protection and lubrication of the cornea. However, many other functions have elaborated and they demonstrate much species variation. In rodents, a few of these additional functions include the secretion of pheromones that influence social interactions (Payne, 1977), cushioning the eye, possible involvement in extraretinal photoreception in the neonatal rat (Wetterberg *et al.*, 1970), and the Harderian gland secretions coat the hair and facilitate thermoregulation in gerbils (Thiessen and Kittrell, 1980).

The effect of nitrofen on the growth of the mice in the present study was also unusual in that the growth rates were normal during the first few weeks after birth at doses of 25 mg/kg and lower but were subsequently retarded in the 12.5 and 25 mg/kg/day dosage groups. The effect on growth rate was more easily detected as the animals matured as was detection of the Harderian gland defect. Interestingly, thyroid hormones are not necessary for fetal growth but they are critical for postnatal development (D'Ercole and Underwood, 1981).

In addition to these effects, prenatal nitrofen treatment also retarded the growth of lungs, an observation made by other investigators (Stone and Manson, 1981; Kavlock *et al.*, 1982). Our results indicate that lung development was more retarded in the 35-day-old males than in 110-day males (significant dose by age interaction, $p < 0.01$), suggesting that the lungs of the treated mice were recovering, in part, from their underdeveloped condition. Maturation of lungs during the perinatal period is also impaired by thyroidectomy (Erenberg *et al.*, 1979) and accelerated with thyroxine injections (Wu *et al.*, 1973). Maturation of the lung is also dependent upon glucocorticoids, and an alteration

TABLE 7

A SUMMARY OF THE EFFECTS OF PRENATAL NITROFEN EXPOSURE ON THE POSTNATAL DEVELOPMENT OF THE CD-1 MOUSE

Dose of nitrofen listed below is the lowest effect level for the variables on the right (mg/kg/day)	Variables significantly affected at the dose to the left
6.25	Harderian gland weight reduction; liver weight reduction; lung weight at 35 and 110 days of age reduced
12.5	Seminal vesicle weight reduction; body weight at 110 days of age reduced
25	Harderian gland ablation (4%); reduced palpebral fissure height
50	Reduced palpebral fissures on Day 30; Harderian gland ablation (65%); delayed puberty in females; lowered fertility
100	Paired testes weight reduced; reduced viability of pups from Day 3 to 30; No. of live pups and body weight on Day 3 reduced
150	No. of live pups on Day 1 reduced, and by Day 3 all pups dead
200[a]	Anophthalmia; hydrocephaly; hyperactivity; malocclusion of jaws

[a] Results are from an earlier study where the dams were dosed with nitrofen on Days 8 to 12 of gestation.

of the developmental hormonal milieu can result in respiratory distress (RDS) in the newborn (Hitchcock *et al.*, 1980). Although the mechanism by which nitrofen affects the Harderian glands, retards lung development, and produces permanent stunting was not determined in the present study, it is interesting that the development of these organs is dependent upon normal thyroid function (Wetterberg *et al.*, 1970; Hitchcock *et al.*, 1980; Bakke *et al.*, 1976) and leads us to speculate that some of the teratogenic effects of nitrofen may be related to an alteration of the thyroid physiology of the perinate.

Although the hypothesis that nitrofen alters fetal development via an alteration of thyroid function is speculative, it is clear that nitrofen interferes with the thyroid physiology of adult rodents. A National Cancer Institute study noted that chronic dietary administration of nitrofen in rats resulted in an increase in cellular hyperplasia in the thyroid (U.S. Department of Health, Education and Welfare, 1979), and acute exposure to nitrofen lowers serum thyroxine levels in adult female mice (unpublished data). Interestingly the thyroid hormones and nitrofen are all halogenated diphenyl ethers. Similarly, polyhalogenated biphenyls, including PCBs and PBBs, cause hypothyroidism in rodents by increasing thyroxine excretion (Barsano, 1981).

Prenatal nitrofen treatment caused abnormalities of reproductive development in addition to its effects on body, lung, and Harderian gland weights. The reproductive development of male, as indicated by testis and seminal vesicle weight reductions, and female mice, as measured by the age of vaginal opening, was retarded by prenatal nitrofen treatment at 50 and 100 mg/kg. In addition, the breeding pairs composed of males and females from the 50 and 100 mg/kg/day dose groups had smaller litters.

Although Stone and Manson (1981) reported that prenatal exposure to nitrofen caused severe hydronephrosis in rats, this effect was not seen in the present study with mice. We have also seen the severe hydro-

nephrosis described by these authors in our studies on rats and hamsters. The lack of an affect on the kidney in the present study may represent a species difference between these rodents in the sensitivity of this organ.

The rank order of the sensitivities of the other measured variables to prenatal nitrofen exposure is shown in Table 7. It is important to note that although the effects noted in the 6.25 mg/kg dose group are small, a multivariate analysis of the organ weights, excluding the Harderian glands, indicates that the noted differences between the 0 and 6.25 mg/kg dose groups would occur in only 2 of 10,000 experiments by chance alone.

Although a wide variety of developmental defects were noted in the present study, these abnormalities were either not present or not readily detectable at birth but became apparent as these systems matured later in life. Standard teratology testing, which examines late-term fetuses for morphological defects, or neonatal pups, as in the present study, underestimates the teratogenic potential of the herbicide nitrofen. Necropsy of the dying or dead pups in the present study and the observation of fetal mice in a concurrent study revealed a low incidence of gross morphological defects at the 100 mg/kg/day dosage group and above, and the incidence of frank terata at the 150 and 200 mg/kg/day dosage groups was not sufficient to predict the complete mortality of these pups after birth. We believe that the present postnatal evaluation after gestational exposure to nitrofen provides essential information for the evaluation of its teratogenic potential and demonstrates the utility of this type of testing.

REFERENCES

AMBROSE, A. M., LARSON, P. S., BORZELLECA, J. F., SMITH, R. B., JR., AND HENNIGAN, G. R., JR. (1971). Toxicologic studies on 2,4-dichlorophenyl-*p*-nitrophenyl ether. *Toxicol. Appl. Pharmacol.* **19**, 263–275.

BAKKE, J. L., LAWRENCE, N. L., ROBINSON, S., AND BENNETT, J. (1976). Life-long alterations in endocrine function resulting from brief perinatal hypothyroidism in the rat. *J. Lab. Clin. Med.* **88**, 3–13.

BARSANO, C. P. (1981). Environmental factors altering thyroid function and their assessment. *Environ. Health Perspect.* **38**, 71–82.

BOAS, N. F., AND SCOW, R. O. (1954). Apparent exophthalmos in the rat following cortisone treatment or thyroidectomy. *Endocrinology* **55**, 148–155.

CHERNOFF, N., AND KAVLOCK, R. J. (1980). A potential *in vivo* screen for the determination of teratogenic effects in mammals. *Teratology* **21**, 33A.

COSTLOW, R. D., AND MANSON, J. M. (1981). The heart and diaphragm—Target organs in the neonatal death induced by nitrofen (2,4-dichlorophenyl-*para*-nitrophenyl ether). *Toxicology* **20**, 209–228.

COURTNEY, K. D., AND CHERNOFF, N. (1974). *Training Manual for Teratology*, p. 13. U.S. Environmental Protection Agency (EPA-650/1-73-701).

D'ERCOLE, A. J., AND UNDERWOOD, L. E. (1981). Growth factors in fetal growth and development. In *Fetal Endocrinology* (M. J. Novy and J. A. Resko, eds.), pp. 155–182. Academic Press, New York.

DOHLER, K. D., WONG, C. C., AND VON ZUR MUHLER, A. (1979). The rat as a model for the study of drug effects on thyroid function: Consideration of Methodological problems. *Pharmacol. Ther.* **5**, 305–318.

ERENBERG, A., RHODES, M. L., WEINSTEIN, M. M., AND KENNEDY, R. L. (1979). The effect of fetal thyroidectomy on ovine fetal lung maturation. *Pediatr. Res.* **13**, 230–235.

GRAY, L. E., JR., KAVLOCK, R. J., CHERNOFF, N., FERRELL, J., LAMB, J., AND OSTBY, J. (1982). Prenatal exposure to the herbicide 2,4-Dichlorophenyl-*p*-nitrophenyl Ether destroys the rodent Harderian gland. *Science* **215** (4530), 293–294.

HELWIG, J. T., AND COUNCIL, K. A. (1979). *SAS User's Guide*, 1979 ed. SAS Institute, Cary, N.C.

HITCHCOCK, K. R., HARVEY, J., AND REICHLIN, S. (1980). Hormones and the lung. I. Thyroid hormone uptake kinetic of perinatal rat lung. *Endocrinology* **107**, 294–299.

KAVLOCK, R. J., CHERNOFF, N., ROGERS, E., WHITEHOUSE, D., CARVER, B., GRAY, J., AND ROBINSON, K. (1982). An analysis of fetotoxicity using biochemical endpoints of organ differentiation. *Teratology* **26**, 183–194.

KENNEDY, G. Y. (1970). Harderoporphyrin: A new porphyrin from the Harderian glands of the rat. *Comp. Biochem. Physiol.* **36**, 21–36.

KIMBROUGH, R. D., GAINES, T. B., AND LINDER, R. E. (1974). 2,4-Dichlorophenyl-*p*-nitrophenyl ether (TOK): Effects on lung maturation of rat fetus. *Arch. Environ. Health* **28**, 316–320.

NAKAO, Y., VEKI, R., TADA, T., IRITANI, I., AND KISHIMOTO, H. (1981). Experimental animal model of congenital diaphragmatic hernia induced chemically. *Teratology* **24**, 11A.

PAYNE, A. P. (1977). Pheromonal effects of Harderian gland homogenates on aggressive behavior in the hamster. *J. Endocrinol.* **73**, 191–192.

REITER, L., ANDERSON, G., ASH, M., AND GRAY, L. E., JR. (1977). Locomotor activity measurements in behavioral toxicology: Effects of lead administration on residential maze behavior. In *Behavioral Toxicology: An Emerging Discipline*, EPA-600/9-77-042 (H. Zenich and L. Reiter, eds.), pp. 6-1–6-18, USEPA, Research Triangle Park, N.C.

STONE, L. C., AND MANSON, J. M. (1981). Effects of the herbicide 2,4-dichlorophenyl-*para*-nitrophenyl ether (Nitrofen) on fetal lung development in rats. *Toxicology* **20**, 195–208.

THIESSEN, D. D., AND KITTRELL, M. W. (1980). The Harderian gland and thermoregulation in the gerbil (*Meriones unguiculatus*). *Physiol. Behav.* **24**, 417–424.

U.S. Department of Health, Education and welfare. (1979). *Bioassay of Nitrofen for Possible Carcinogenicity.* Publication No. (NIH) 79-1740. CAS No. 1836-75-5, NCI-CG-TR-184.

WETTERBERG, L., UWILER, A., GELLER, E., AND SHAPIRO, S. (1970). Harderian gland: Development and influence of early hormonal treatment. *Science* **168**, 966–998.

WU, B., KIKKAWA, Y., ORZALESI, M. M., MOTOYAMA, E. K., KAIBARA, M., ZIGAS, C. J., AND COOK, C. D. (1973). The effect of thyroxine on the maturation of fetal rabbit lungs. *Biol. Neonate* **22**, 161–168.